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1    **The isopod *Eurydice spinigera* and the chaetognath *Flaccisagitta enflata*: how the habitat**  
2    **affects bioaccumulation of metals in predaceous zooplankton**

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12  
13    Bioaccumulation processes result in a high enrichment of metals in zooplankton communities. In  
14    terms of trace element concentrations, most information involves copepods, which are often the  
15    dominant forms in the community, but are not the only common zooplankton species.

16    We analyzed the concentrations of 20 trace elements in *Eurydice spinigera* (Isopoda) and  
17    *Flaccisagitta enflata* (Chaetognata), which represent two different species of marine zooplankton  
18    that share the same feeding predaceous strategy, from a highly productive coastal region (Ligurian  
19    Sea, Northwestern Mediterranean).

20    Our results demonstrated that metal transfer was deeply influenced by the different habitats, as the  
21    carnivorous isopod *E. spinigera*, which spends most of its lifetime on the seabed, had the highest  
22    concentrations of most of the analyzed trace elements (Al, As, Cd, Ce, Cr, Fe, La, Mn, Ni, Pb, Se,  
23    V and Zn) and consequently the highest bioaccumulation factors (BAFs). Conversely, in the  
24    carnivorous Chaetognatha *F. enflata*, which is not a benthonic species, the highest levels of copper  
25    and tin were found. Moreover, arsenic speciation analysis confirmed the presence of inorganic As  
26    (III+V) in *E. spinigera*. In the perspective of utilizing a marine organism as a bio-indicator of metal  
27    transfer, it is crucial to consider both feeding behavior and feeding habitat.

28

29

30    **KEYWORDS:** trace elements, zooplankton, hyperbenthos, chaetognaths, isopods, Mediterranean

31    Sea.

## 32 INTRODUCTION

33 In the last decade, there has been a considerable interest in understanding metal accumulation in  
34 marine organisms as they can greatly influence the cycling, fluxes, and residence times of metals in  
35 marine systems. Aquatic invertebrates are exposed to chemicals from both the particulate and  
36 dissolved phases, and play a key role in the trophic transfer of metals in aquatic food chains as food  
37 uptake has been increasingly recognized as an important source for accumulation of contaminants  
38 (Fisher and Reinfelder, 1995). Zooplankton is particularly critical to the functioning of ocean food  
39 webs due to their abundance and their vital ecosystem roles. In assessing environmental quality  
40 with respect to trace elements in seawater, the bioavailable fraction is of major importance as  
41 toxicity depends on the bioavailable exposure concentration (Kahle and Zauke, 2003). This  
42 bioavailable fraction can be assessed by determining the amount of metals incorporated into  
43 organisms, which is the main goal in biomonitoring (Rainbow, 1993). Bioaccumulation, along with  
44 persistence and acute toxicity, can be used for identifying aquatic environmental hazards in order to  
45 determine the potential for adverse effects to biota (McGeer *et al.*, 2003). The BAF  
46 (Bioaccumulation Factor) is a model for bioaccumulation that predicts partitioning between an  
47 exposure medium (marine water) and biota (zooplankton species), and is calculated as the ratio of  
48 internal biota concentration to exposure concentration (McGeer *et al.*, 2003).

49 The order Isopoda is a ubiquitous monophyletic *taxon* that includes around 10,131 species (Boyko  
50 *et al.*, 2008) found in all ecosystems from the deepest oceans to the montane terrestrial habitats and  
51 deep underground in caves or aquifers. The most significant feature of the group is the  
52 diversification into a number of different ecological roles or modes of life (Argano and Campanaro,  
53 2010), and isopod representatives occur in the marine environment from the littoral to abyssal zones  
54 (Naylor, 1972).

55 Isopods of the family Cirolanidae dominate the upper shore of sandy beaches in most temperate and  
56 tropical regions (Bruce, 1986). Isopods belonging to the genus *Eurydice*, which are highly  
57 predaceous carnivores, particularly including copepods and cladocerans (Macquart-Moulin, 1998),  
58 have pelagic phases during the night (Macquart-Moulin, 1992; Macquart-Moulin and Patrìti, 1996).  
59 The circadian rhythm stimulates them to emerge from the sediment at dusk (Macquart-Moulin,  
60 1973, 1976), i.e. an endogenous light-controlled vertical migration occurs (Macquart-Moulin, 1972,  
61 1985) and the animals gather at the sea surface (Champalbert and Macquart-Moulin, 1970; Tully  
62 and O'Ceidigh, 1986, 1987; Macquart-Moulin, 1992; Macquart-Moulin and Patrìti, 1996). The  
63 hyponeustonic pattern of distribution is observed throughout the night, and at dawn the animals

64 return to the bottom and burrow into the sediment (Macquart-Moulin, 1998). The upward evening  
65 migration and the downward morning migration are both very fast, and only a few specimens have  
66 been recorded in the deep or intermediate layers during the night (Macquart-Moulin, 1998). This  
67 nocturnal migratory behavior occurs close inshore along the whole continental shelf. The migratory  
68 behavior of several *Eurydice* spp., included *E spinigera*, may constitute a mechanism for directly  
69 ensuring active vertical transfer of organic matter and trace elements between the bottom, the  
70 surface and the various water masses along the whole continental margin, including the shelf and  
71 the slope regions.

72 The relatively small isolated *phylum* Chaetognatha, also known as arrow worms, includes a total of  
73 209 species that have been recorded in the world's oceans, of which 20 (16 planktonic and 4  
74 benthic,) have been reported in the Mediterranean Sea (Furnestin, 1979; Bieri, 1991; Kehayias *et*  
75 *al.*, 1999; Ghirardelli, 2010). Exclusively predaceous, chaetognaths are found in marine habitats  
76 including estuaries, open oceans, tide pools, polar waters, marine caves, coastal lagoons and deep  
77 sea waters (Bone *et al.*, 1991). Moreover, chaetognaths are distributed from the surface to great  
78 depths, while some species exclusively live close to the sea floor (Pierrot-Bults and Nair, 1991).  
79 The abundance of chaetognaths is often second only to copepods in the zooplankton of many  
80 marine environments (Feigenbaum and Maris, 1984; Shannon and Pillar, 1986; Gibbons, 1992).  
81 The biomass of chaetognaths is estimated to be 10-30% of that of copepods in the pelagic realm;  
82 thus, they play a significant role in the transfer of energy from copepods to higher trophic levels  
83 (Bone *et al.*, 1991; Feigenbaum, 1991; Froneman *et al.*, 1998; Giesecke and González, 2004). The  
84 diet of chaetognaths includes a variety of pelagic organisms, consisting mainly of copepods, but  
85 they may also prey on larvaceans, cladocerans and fish larvae, thus strongly influencing the  
86 zooplankton and ichthyoplankton communities (Faigenbaum, 1991; Casanova, 1999; De Souza *et al.*,  
87 2014). Inter- and intra-specific predation has been reported among various species of chaetognaths  
88 (Pearre, 1982). Prey selectivity is most often attributed to the prey size (Pearre, 1982), but factors  
89 such as prey swimming behavior, conspicuousness and availability, may also be significant (Duró  
90 and Saiz, 2000; Coston-Clements *et al.*, 2009). Moreover, chaetognaths are prey to many larger  
91 organisms including fishes, whales, other marine invertebrates and molluscs. Diel vertical migration  
92 (DVM), is common among chaetognaths (Terazaki, 1996; Giesecke and González, 2004; Johnson *et*  
93 *al.*, 2006; Kehayias and Kourouvakalis, 2010). However, most studies on chaetognath DVM have  
94 been conducted in areas with water depths exceeding 50 m, while studies in shallow waters are  
95 scarce (Sweatt and Forward, 1985).

96 Chaetognaths have another characteristic that makes them particularly interesting from an  
97 oceanographic point of view. They have been shown to be good indicators of water masses (Pierrot-  
98 Bults, 1982; Ulloa *et al.*, 2000; Kehayias *et al.*, 2004) and, consequently, appear to be very suitable  
99 for studying the effects of physical processes- acting at the mesoscale- on the dynamics and  
100 variability of zooplankton populations (Duró and Saiz, 2000). Ecologically, *Flaccisagitta enflata*  
101 predominates in the tropical-subtropical epipelagic waters (Pierrot-Bults and Nair 1991; Duró and  
102 Saiz, 2000). *F. enflata* is adapted to the uppermost layers of the warm-water sphere, which has the  
103 lowest density and a vertical range of only 100 m to 200 m (Kapp 1991); numerically, it is the most  
104 important chaetognath neritic species of the Mediterranean Sea (Batistic, 2003; Ghirardelli and  
105 Gamulin, 2004). In the Northwestern Mediterranean Sea, the main copepod prey of *F. enflata* was  
106 reported to be *Centropages typicus* and *Temora stylifera* (Duró and Saiz, 2000). Recently,  
107 bioaccumulation of trace elements in chaetognaths belonging to the family Sagittidae, was  
108 investigated in the coastal regions of India (Bhattacharya *et al.*, 2014), and in the White Sea (Budko  
109 *et al.*, 2015).

110 Secondary consumers in zooplankton communities, Isopoda and Chaetognata could be suitable  
111 “indicators” of the presence and transfer of metals in a marine environment. Bioindicators are  
112 organisms- a particular species or communities of species- used to assess the quality of an  
113 environment or changes in the environment due to anthropogenic disturbances or natural stressors.  
114 In a previous study, we analyzed the potentiality of marine zooplankton to be bioindicators of trace  
115 elements in coastal ecosystems (Battuello *et al.*, 2016). We found that the examined zooplankton  
116 showed a great ability to accumulate concentrations of metals that were several thousand times  
117 more than concentrations detected in marine water, in particular the essential elements iron, copper,  
118 zinc, cobalt and manganese and the nonessential element cadmium. We then focused on the  
119 influence of the different feeding modes (herbivorous, omnivorous and carnivorous) in metal  
120 bioaccumulation in Calanoida copepods (Battuello *et al.*, 2017), and we found that there was a  
121 reduced metal accumulation in carnivores compared to herbivores. In fact, the herbivorous species  
122 showed the highest concentrations and BAFs for most of the analyzed metals, in particular for the  
123 nonessential elements aluminum and cadmium, and for the essential trace elements copper, iron,  
124 manganese and zinc. Nevertheless, not all species or communities can serve as successful  
125 bioindicators, and expanding on this topic, our study focused on the influence of different habitats  
126 in metal bioaccumulation in two carnivorous zooplankton species: *Eurydice spinigera* (Isopoda)  
127 and *Flaccisagitta enflata* (Chaetognata). In fact, both these species have the potential to be sentinel  
128 species of a marine environment, being able to accumulate and concentrate metals to measurable

129 levels above those in the surrounding waters; in addition, they share the same feeding strategies as  
130 they are both carnivores, and they are both at the top of the zooplankton food web. However, they  
131 have different habitat requirements, and we therefore postulated that the habitat could be significant  
132 in the bioaccumulation of metals through the marine food chain.

133 The main objectives of the present study were:

- 134 i) to analyze, for the first time, the concentrations of 20 trace elements in two zooplanktonic  
135 marine species, *E. spinigera* (Isopoda) and *F. enflata* (Chaetognata)
- 136 ii) to evaluate the relevance of these two predaceous species in the bioaccumulation and  
137 transfer of trace elements through the marine food chain
- 138 iii) to establish the suitability of *E. spinigera* and *F. enflata* as bioindicators of different  
139 compartments of a marine coastal environment.

## 140 **METHODS**

### 141 **Study area and sampling site**

142 The study area is a highly productive Italian coastal region characterized by heavy commercial  
143 maritime traffic and numerous industrial plants. Indeed, the area currently has one of the highest  
144 levels of shipping in the whole Mediterranean basin, and is a recipient of pollutants coming from  
145 the highly developed coastline of Italy. Furthermore, this coastal area also experiences summer  
146 tourism, which leads to a substantial increase in inhabitants and consequently to elevated risks of  
147 pollution (Barrier, 2016). As a result, municipal wastewater treatment plants show effluents  
148 characterized by a lower water quality and an increase in the nutrient concentration of marine water  
149 (Renzi *et al.*, 2010). The sampling site was situated off the Italian coast, in the transition zone  
150 between the Northern Tyrrhenian Sea and the Southern Ligurian Sea (Fig. 1). The sampling station  
151 (43°28'10" N, 10°01'55" E) was located at 12.5 nm off the Tuscan coast, above the continental  
152 shelf. The sector under investigation is characterized by a large extension of the continental shelf  
153 and limited depth (100 m), even at considerable distances from the coast (18 miles) (Chiocci and La  
154 Monica, 1996). The Ligurian Sea lies at the north-east edge of the Western Mediterranean and is  
155 connected to the southern basin (Tyrrhenian Sea) via the Corsica Channel. The general circulation  
156 of the Ligurian Basin is characterized by a permanent basin-wide cyclonic circulation involving  
157 both the surface Modified Atlantic Water (MAW) and the lower Levantine Intermediate Water  
158 (LIW) (Millot, 1999; Bozzano *et al.*, 2014). The Northern Current is generally weaker in the  
159 summer than during the winter and the contribution from the Tyrrhenian Sea is strongly reduced in  
160 summertime (Aliani *et al.*, 2003). The flow originates before the Ligurian Sea due to the merging of  
161 the Western and Eastern Corsican Current through the Corsica Channel (Artale *et al.*, 1994).

162 Climatic forcing can greatly change the intensity of currents, but the general pattern can be  
163 considered permanent (Molinero *et al.*, 2005; Birol *et al.*, 2010). Moreover, due to the interplay of  
164 these particular oceanographic, climatic and physiographic factors, the area is highly productive and  
165 hosts a rich and complex ecosystem. This is also sustained by vertical mixing and coastal  
166 upwelling, generated by the prevailing northwesterly wind, which pumps nutrients and other  
167 organic substances contributed by rivers into the euphotic zone where they fertilize growing  
168 phytoplankton populations (Bozzano *et al.*, 2014). Hence, the area attracts several cetacean species  
169 and is part of the “Cetacean Sanctuary” protected area.

## 170 **Sampling**

171 Zooplankton samples were collected during September 2015 (summer). The sampling station was  
172 located on the continental shelf above a bottom depth of 111 m (Fig.1). Surface zooplankton  
173 samples were caught with a WP-2 standard net, having a mesh size of 200  $\mu\text{m}$  and a diameter of 57  
174 cm. The net was towed horizontally at the water surface and the sampling time was approximately  
175 15 min at a vessel cruising speed of 2 knots. Each net was fitted with a flow meter (KC Denmark  
176 model 23.090) to measure the volume of water filtered, which ranged from 251.63 to 329.2  $\text{m}^3$ . Net  
177 hauls were consistently carried out at night to allow surface sampling of isopods and chaetognaths,  
178 involved in nocturnal migrations.

179 The entire sample from each net was divided into two aliquots immediately after sampling, using a  
180 Folsom splitter. One aliquot was fixed in 4% neutralized formaldehyde buffered with borax and  
181 kept in the dark, for analyzing the zooplankton composition, with particular attention to the  
182 identification and quantification of dominant isopod and chaetognath species (Boltovskoy, 1981).  
183 The second aliquot was also immediately fixed, in the same manner, for subsequent analysis of  
184 trace element concentrations of target species (Fang *et al.*, 2014; Fernandez de Puelles *et al.*, 2014).  
185 To avoid possible contaminations on the surface of the zooplanktonic organisms, each sample was  
186 washed four times with distilled water for elimination of fine particulates and kept frozen for trace  
187 metals analyses. In order to quantitatively analyze the trace metals contained in the isopods and  
188 chaetognaths, and compare the differences in metal content relative to their different distribution  
189 and feeding behavior within the water column, samples were sorted, and the selected species were  
190 analyzed for trace metal determination. Regarding the chaetognaths, only adult specimens with  
191 empty guts were taken.

192 Depending on the size and abundance of the different target species, about 300 – 600 specimens of  
193 the two target species were selected separately for each of the four samples. Shallow seawater  
194 samples for total dissolved trace metal analysis were collected at a depth of 1 m using 5 L Niskin



bottles and stored in a cool box until being subjected to filtration. All samples were kept under refrigerated conditions before analysis.

#### **Detection of trace elements**

*E. spinigera* and *F. enflata* samples (n=4 for each species) were accurately rinsed with Milli-Q water to remove the formaldehyde buffer before trace elements quantification.

Determination of aluminum (Al), arsenic (As), beryllium (Be), cadmium (Cd), cerium (Ce), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), lanthanum (La), lead (Pb), nickel (Ni), antimony (Sb), selenium (Se), tin (Sn), (thallium) Tl, vanadium (V) and zinc (Zn) was performed after wet digestion using acids and oxidants (HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub>) of the highest quality grade (Suprapure). Samples were subjected to microwave digestion (microwave oven ETHOS 1 from Milestone, Shelton, CT, USA) with 7 mL of HNO<sub>3</sub> (70% v/v) and 1.5 mL of H<sub>2</sub>O<sub>2</sub> (30% v/v). Ultrapure water was added to samples to reach a final weight of 50 g (Arium611VF system from Sartorius Stedim Italy S.p.A., Antella - Bagno a Ripoli, FI, Italy). All metals were quantified by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany). Multi-elemental determination was performed after daily optimization of instrumental parameters, and use of an external standard calibration curve; rhodium and germanium were used as internal standards. Analytical performances were verified by processing Certified Reference Materials (Oyster Tissue -SRM 1566b from the National Institute of Standard and Technology), along with blank reagents in each analytical session. The limit of quantification (LOQ) for each element, the reference material values and the percentages of recovery obtained are shown in Table S1.

A chelating polymer resin, the SPR-IDA Reagent (Suspended Particulate Reagent – Iminodiacetate, by Cetac Technologies, Omaha, USA) was used for pre-concentration/ matrix elimination of seawaters. A volume of 15 mL of seawater was directly added to a pre-cleaned 15 mL volume polypropylene centrifuge tube. A 100-μL aliquot of a 10% suspension of SPR-IDA reagent beads was then pipetted directly onto the sample. Tubes were covered with parafilm and contents were mixed thoroughly. Samples were then spiked with 0.5 μg L<sup>-1</sup> yttrium, which functions as an internal standard, in order to correct for any volume differences in the blanks, samples, and spiked samples. High-purity ammonium hydroxide (NH<sub>4</sub>OH, 29%) was added in two steps (25 μL + 20 μL) to adjust the pH to approximately 8. The SPR-IDA beads were then allowed to settle for approximately 1 h. Samples were then placed in a centrifuge and spun at 2000 rpm for 10 min. The supernatant liquid was carefully poured off to minimize any loss of beads, which were mostly compacted at the bottom of the tube. A solution of deionized water, adjusted to pH 8 with high

228 purity NH<sub>4</sub>OH, was then added to the 15 mL mark of the sample tube and the contents were mixed.  
229 The beads were again allowed to settle, centrifuged, and the resulting supernatant liquid was  
230 carefully poured off and discarded. A 0.5 mL aliquot of 7% v/v absolute high-purity nitric acid  
231 (Suprapure) was then added to the bead residue to extract any bound metal ions. The resulting  
232 extract was diluted to 3 mL with deionized water and analyzed by ICP-MS. The following metals  
233 were then quantified in seawater: Al, Cd, Co, Cu, Fe, Mn, Ni, Pb, Zn.

#### 234 **Arsenic speciation**

235 Arsenic speciation analysis was conducted by high performance liquid chromatography coupled to  
236 an inductively coupled plasma mass spectrometer (HPLC-ICP-MS); ICP-MS Xseries II, Thermo  
237 Scientific, Bremen, Germany and HPLC Spectra System MCS 1000, Thermo Scientific, Bremen,  
238 Germany) following the Thermo Scientific application note n° 40741.

239 The following As species were investigated: DMA (Dimethylarsinic acid), MMA  
240 (Monomethylarsonic acid), AsB (Arsenobetaine), iAs (sum of As III, arsenite, and As V, arsenate).  
241 The limit of quantitation of the method (LOQ) was 0.020 mg Kg<sup>-1</sup> for all the arsenical species.

#### 242 **Bioaccumulation factors (BAFs)**

243 The bioaccumulation factor (BAF) is the ratio of the concentration of a chemical in an organism  
244 compared to the concentration in water. BAFs were estimated for Al, Cd, Co, Cu, Fe, Mn, Ni, Pb  
245 and Zn, the same elements that were quantified in both seawater and in the analyzed species.  
246 For estimating BAFs, the metal levels were expressed as µg kg<sup>-1</sup> in *E. spinigera* and *F. enflata* as  
247 µg L<sup>-1</sup> in seawater.

#### 248 **Statistical analysis**

249 All statistical analyses were performed within the R statistical framework (R Core Team, 2015).  
250 Normality of data and equality of variance were assessed. The abundance of *E. spinigera* and *F.*  
251 *enflata*, as well as the whole mesozooplanktonic assemblages from each replicate sample was  
252 compared using the Kruskal-Wallis test ( $p < 0.05$  was considered as statistically significant), to  
253 determine if it was acceptable to combine these datasets. There were no significant differences  
254 between the four replicates (Kruskal-Wallis  $X^2 = 1.550$ ,  $df = 3$ ,  $p = 0.671$ ).

255 Before analyzing metal concentrations, we performed the D'Agostino-Pearson normality test to  
256 determine the distribution of the values. The unpaired t test was used to test differences in metal  
257 concentrations between the isopod and the chetognath. Results were considered statistically  
258 significant with p values of  $< 0.05$ . Statistical calculations were performed using Graph Pad  
259 Statistics Software Version 6.0 (GraphPad Software, Inc., USA).

260

261

262 **RESULTS AND DISCUSSION**

263

264 **Zooplankton communities**

265 The mesozooplankton communities of the replicates showed comparable compositions, so we  
 266 presented their mean values to facilitate data discussion. Copepods made up the bulk of the  
 267 zooplankton biomass (74.36%), with 61.21% of the total biomass comprised of calanids  
 268 (Calanoida). Cladocerans were the second largest group (9.47%), followed by chaetognaths  
 269 (3.21%), pteropods (3.03%), larvaceans (2.42%), siphonophores (2.11%), euphausiids (2.05%),  
 270 isopods (1.84%), mysidaceans (1.30%), and ostracods (0.21%). Overall, the zooplankton collected  
 271 in summer 2015 presented a mean biomass value of 2.57 mg m<sup>-3</sup> (expressed as dry weight,  
 272 Lovegrove, 1966). Chaetognaths were represented by three species, namely the dominant neritic  
 273 *Flaccisagitta enflata* (3.622 ind.m<sup>-3</sup> ± 2.417), the epiplanktonic *Mesosagitta minima* (1.763 ind.m<sup>-3</sup>  
 274 ± 0.335) and *Parasagitta friderici* (0.134 ind.m<sup>-3</sup> ± 0.018), a neritic species. Only two isopod  
 275 species were recorded: the hyperbenthic *Eurydice spinigera* (Cirolanidae, 1.030 ind.m<sup>-3</sup> ± 0.011)  
 276 and the neustonic *Idotea metallica* (Idoteidae, 0.031 ind.m<sup>-3</sup> ± 0.007).

277 **Trace elements in seawaters**

278 The concentrations of dissolved Mn, Fe, Cu, Zn, Al, Ni Co, Cd and Pb are shown in Fig. S1 and are  
 279 reported as µg L<sup>-1</sup>. In surface water, metal concentrations were found in the following order:  
 280 Zn>Ni>Fe>Al>Pb>Co>Mn>Cu>Cd; i.e. Zn and Ni were the nutrient trace elements with the  
 281 highest concentrations, 11.43 and 10.60 µg L<sup>-1</sup>, respectively. The level of the nonessential element  
 282 Al was relatively low (1.50 µg L<sup>-1</sup>) and in line with previous findings in the Mediterranean Sea  
 283 (Caschetto and Wollast, 1979; Battuello *et al.*, 2016). Trace metals in coastal waters are usually  
 284 higher than concentrations in the open ocean, owing to metal influx from continental sources, such  
 285 as ground water and coastal sediments (Sunda, 2012). The concentrations that were detected in  
 286 seawater were comparable or lower than those recently detected in the Mediterranean Sea (Safaa,  
 287 2015; Ebling and Landing 2015; Battuello *et al.*, 2016). The metal concentrations in water does not  
 288 provide information on metal bioaccumulation or biomagnification in biota (Ricart *et al.*, 2010;  
 289 Maceda-Veiga *et al.*, 2013) but it is necessary to estimate the bioaccumulation factors.

290 **Trace elements in *E. spinigera* and *F. enflata***

291 The concentration of trace elements (Fig. 2 and 3) was in the following order:  
 292 Zn>Cu>Al>Fe>Mn>Pb>Ni>Se>Cd>Cr>As>Co>V>Ce>Sn>Mo>La>Sb in the isopod *E. spinigera*  
 293 and Zn>Cu>Fe>Al>Ni> Mn>Pb>Se>Cr>Sn>As>Co>V>Cd>Mo=Sb>Ce>La in the chaetognath *F.*

294 *enflata*. In Table 1 descriptive statistics were shown for each species samples. The statistical  
295 evaluation results are shown in Table 2.

296

#### 297 *Essential trace elements*

298 Isopods had the highest values for all the essential trace elements (cobalt, chromium, iron,  
299 manganese, molybdenum, nickel, selenium and zinc), with the exception of copper, which was  
300 higher in chaetognaths, and molybdenum, which was the same concentration in both species.

301 Statistically significant differences were found between the two species for all the essential  
302 elements (Table 2).

303 Manganese and zinc values were much higher in isopods than in chaetognaths (Table 1, Table 2,  
304 Fig. 2). Mn is a naturally occurring metal in seawater, and it is well known that it can be  
305 significantly bioconcentrated by aquatic biota at lower trophic levels (WHO, 2004); Mn  
306 concentration was an order of magnitude higher in *E. spinigera* (4.40 mg Kg<sup>-1</sup>) than in *F. enflata*  
307 (0.49 mg Kg<sup>-1</sup>).

308 Zinc is essential for the biological requirements of marine plankton, and its concentration usually  
309 greatly exceeds that required for normal metabolism in tissues of aquatic organisms because it  
310 concentrates more effectively than other elements. Accordingly, Zn was the most represented  
311 element in both species, but its concentration was much higher in *E. spinigera* (234.02 mg Kg<sup>-1</sup>)  
312 than in *F. enflata* (98.12 mg Kg<sup>-1</sup>). These essential elements, Mn and Zn, showed nutrient-like  
313 vertical distributions, being depleted in surface waters due to uptake by the biota, and increased in  
314 concentration with increasing depths, because of the remineralization of sinking organic matter  
315 (Sunda, 2012). This is consistent with the higher concentrations of Mn and Zn observed in the  
316 benthonic species *E. spinigera*.

317 Copper was higher in chaetognaths (84.60 mg Kg<sup>-1</sup>) than in isopods (53.15 mg Kg<sup>-1</sup>) possibly  
318 indicating a higher availability of this element in the upper water column. It is well known that  
319 marine organisms are able to concentrate significant amounts of copper in seawater, which is  
320 required as this element is a component of enzymes and hemocyanin (Paimpillil *et al.*, 2010).  
321 Regarding isopods, and crustaceans in general, the hepatopancreas is the most important storage  
322 organ of heavy metals, containing more than 50% of the total copper in the body (Hopkin *et al.*,  
323 1985; Góral *et al.*, 2009).

324 Iron is an essential element for zooplankton, due to its role in mitochondria of catalyzing redox  
325 reactions during respiration. Marine mesozooplankton can be affected by Fe deficiency in food,  
326 and, due to the role that zooplankton plays in the cycling of Fe and C, these results could have

327 implications for biogeochemical cycles (Chen, 2011). Moreover, a low Fe content in Fe-limited  
328 phytoplankton seems to cause physiological stress in crustacean zooplankton (Chen, 2011). We  
329 found a slightly higher iron content in *E. spinigera* than in *F. enflata* (34.31 and 29.601 mg Kg<sup>-1</sup>,  
330 respectively, Table 1).

331 Chromium, cobalt and selenium concentrations were almost twice as much in *E. spinigera* than in  
332 *F. enflata* (Fig. 2, Table 1). In the open sea, Cr is involved in biogeochemical cycles, with  
333 biologically mediated Cr removal in the surface layers, and elevated Cr levels in deeper waters  
334 because of mobilization of Cr upon breakdown of sinking biogenic particles (Campbell and Yeats,  
335 1981). Nickel is another essential metal for aquatic organisms but it is toxic at elevated  
336 concentrations. There is a relatively high assimilation efficiency and bioavailability of Ni to marine  
337 planktonic organisms (Hutchins and Bruland, 1994); accordingly, we found 1.01 mg kg<sup>-1</sup> of nickel  
338 in *F. enflata* and 1.66 mg kg<sup>-1</sup> in *E. spinigera*.

#### 339 *Nonessential trace elements and rare earth elements*

340 The concentrations of the nonessential trace elements aluminum, arsenic, antimony, cadmium, lead,  
341 tin and vanadium and of the two rare earth elements lanthanum and cerium are shown in Fig. 3.

342 Beryllium and thallium concentrations were undetectable (< LOQ).

343 Differences in nonessential metal levels related to species were statistically significant (Table 2),  
344 apart from antimony.

345 The highest values for all these elements were registered in *E. spinigera*, with the exception of Sn,  
346 which was higher in *F. enflata* and Sb, which differed slightly in the two species.

347 Aluminum was the most represented nonessential trace element in both species, reflecting its  
348 ubiquity in the aquatic environment. The concentration of Al in *E. spinigera* was twice as much as  
349 that in *F. enflata* (38.80 and 15.52 mg kg<sup>-1</sup>, respectively); this agreed with a previous report in  
350 marine zooplankton (Battuello *et al.*, 2016), where higher Al levels were found with increasing  
351 water depths.

352 Seawater naturally contains 1–5 µg L<sup>-1</sup> of total arsenic, which is mainly arsenate and arsenite  
353 (Caunette *et al.*, 2012). Arsenic tends to show a nutrient-like vertical profile in the water column,  
354 indicating biological uptake of arsenic by marine phytoplankton along the phosphate transport  
355 pathway. In addition to inorganic arsenic (iAs), the methylated arsenic species monomethylarsonic  
356 acid (MMA) and dimethylarsinic acid (DMA) are present in water. Phytoplankton accumulates and  
357 methylates the inorganic arsenic (Karadjova *et al.*, 2008), after which phytoplankton organisms are  
358 ingested by zooplankton organisms, which also have other arsenic compounds, such as  
359 arsenobetaine (AsB) (Caumette *et al.*, 2011). We found a total arsenic level of 0.07 mg Kg<sup>-1</sup> in

360 *Flaccisagitta enflata*, and the all arsenical species investigated by As speciation analysis were  
361 undetectable (< LOQ), due to this low content. Interestingly, the isopod *E spinigera* showed a total  
362 As content that was more than three times greater, 0.28 mg Kg<sup>-1</sup>, and a concentration of 0.03 mg  
363 Kg<sup>-1</sup> for the sum of inorganic As species (III+V) was found, while the organic species AsB, DMA  
364 and MMA were < LOQ.

365 The two toxic elements cadmium and lead were an order of magnitude higher in *E spinigera* than in  
366 *F. enflata* (Table 1, Fig.3). Cd has no significant physiological role and it is mainly adsorbed on the  
367 surface of zooplanktonic debris or fecal pellets during its transportation to bottom waters (Kremling  
368 and Pohl, 1989). Pb is known to form colloids in seawater, which can be adsorbed onto planktonic  
369 debris (Paimpillil *et al.*, 2010). We previously observed an increase in Pb concentrations in marine  
370 organisms with increasing water depths (Battuello *et al.*, 2016), and as such, it is not unexpected to  
371 find the highest Pb level (3.07 mg Kg<sup>-1</sup>) in the benthonic species *E spinigera*, which is a level  
372 comparable or lower than previous findings in Mediterranean coastal areas (Rossi and Jamet, 2008).  
373 Similarly, vanadium concentrations were higher in *E. spinigera* in this study and in planktons from  
374 deep waters (Battuello *et al.*, 2016), while tin, which is usually present as organotin compounds in  
375 proximity to harbor areas, as well as to industrial and domestic points of effluent discharge, was  
376 higher in *F. enflata* (Table 1, Fig. 3).

377 Dissolved rare earth elements such as cerium and lanthanum are reported to be present in very low  
378 concentrations in open seawater, typically in the order of pg L<sup>-1</sup> (Wang and Yamada, 2007), but can  
379 be bioaccumulated by marine invertebrates, such as zooplankton, and enter the food chain (Palmer  
380 *et al.*, 2006). Accordingly, we found low Ce and La concentrations in both species (Fig. 3), but the  
381 highest values were registered in the benthonic species *E. spinigera* (0.06 and 0.03 mg Kg<sup>-1</sup>  
382 respectively).

### 383 *Bioaccumulation factors (BAFs)*

384 Given the wide range of concentrations present in the BAF dataset, values were converted to a log  
385 scale to aid visual comparisons (Fig. 4). Our results confirmed the high potential of both species,  
386 particularly *E. spinigera*, to be bioaccumulators of metals.

387 The estimated BAFs were in the following order:

388 *E. spinigera* Cu>Cd>Al> Zn> Fe>Mn> Pb>Ni>Co

389 *F. enflata* Cu>Al> Fe>Zn> Cd> Mn> Pb>Ni>Co

390 BAF trends were fairly similar between the two species, but the BAF order of magnitude was quite  
391 high in the benthonic species, reflecting a greater availability of metals in the deeper waters and in  
392 the seabed sediments. The mechanisms of metal bioaccumulation have been studied in terrestrial

393 isopods, which showed a great capacity to bioaccumulate metals from the environment, especially  
394 copper, which concentrated in the hepatopancreas (Wieser *et al.*, 1977). Accordingly, copper was  
395 the most accumulated trace element in both species, while cadmium was bioaccumulated at  
396 different levels in isopods and chetognaths, being more concentrated in *E. spinigera*, probably  
397 reflecting the vertical distribution of dissolved Cd in ocean waters, characterized by a surface  
398 depletion and deep water enrichment (Boyle *et al.*, 1976).

399 Bioaccumulation of a chemical is affected by rates of uptake, metabolism, and elimination, as well  
400 as the storage capacity of an organism, and several abiotic and biotic factors affect the  
401 bioavailability of metal compounds, e.g. metal speciation, physicochemical parameters of the  
402 environment, and biological–physiological properties of the exposed organism (McGeer *et al.*,  
403 2003). Bioaccumulation itself is not an indicator for a toxic response, since only a certain  
404 proportion of the total internally-accumulated metal concentration- the body burden - may be  
405 metabolically available (Herrmann *et al.*, 2016). However, the bioaccumulation factors clearly  
406 reflect the presence and availability of metals in a determined ecosystem and in different habitats,  
407 confirmed by our study.

#### 408 **Arsenic**

409 Seawater naturally contains 1–5  $\mu\text{g L}^{-1}$  of total arsenic, which is mainly arsenate and arsenite; in  
410 addition to inorganic arsenic (iAs), the methylated arsenic species monomethylarsonic acid (MMA)  
411 and dimethylarsinic acid (DMA) are present in seawater (Caumette *et al.*, 2012). Arsenic tends to  
412 shows a nutrient-like vertical profile in the water column, indicating biological uptake of arsenic by  
413 marine phytoplankton along the phosphate transport pathway. Phytoplankton is able to accumulate  
414 and methylate the inorganic arsenic (Karadjova *et al.*, 2008), and it contains iAs as the majority of  
415 identified arsenic, with methylated arsenic MMA and DMA and arsenosugars as organoarsenic  
416 compounds (Caumette *et al.*, 2012). Zooplankton organisms ingest phytoplankton, and other arsenic  
417 compounds are found in zooplankton, such as arsenobetaine (AsB) (Caumette *et al.*, 2012). Marine  
418 zooplankton contains AsB as a minor compound in herbivorous zooplankton and as a major  
419 compound in carnivorous zooplankton (Shibata *et al.*, 1996). We found a very low total arsenic  
420 level in *Flaccisagitta enflata* ( $0.07 \text{ mg Kg}^{-1}$ ) and the arsenical species that could be detected by As  
421 speciation analysis (water soluble species) were undetectable ( $< \text{LOQ}$ ). Only one study has  
422 investigated the presence of As in Sagittoidea (Shibata *et al.*, 1996, Japan Sea) and found  
423 arsenobetaine to be the dominant arsenic species. The authors suggested that the arsenic compounds  
424 in zooplankton reflect their feeding habit; carnivorous species accumulate arsenobetaine, while  
425 herbivorous species accumulate arsenosugars.

Other studies performed on carnivorous zooplankton, such as amphipods and Antarctic krill collected in the ocean always found arsenobetaine as a major compound (Caumette *et al.*, 2012). Arsenobetaine is described as the only non-toxic arsenic compound and its presence in organisms is assumed to be the result of a detoxification process, but recent studies seem to support the function of AsB in an osmolytic role, suggesting a relationship between salinity and AsB accumulation in marine organisms (Clowes *et al.*, 2004; Larsen and Francesconi, 2003). Interestingly, in our findings, *E. spinigera* showed a total As content ( $0.28 \text{ mg Kg}^{-1}$ ) more than three times higher than *Flaccisagitta enflata* ( $0.07 \text{ mg Kg}^{-1}$ ), and  $0.03 \text{ mg Kg}^{-1}$  of iAs (III+V) was found in the isopod. Among the organic As compounds, traces of MMA were found ( $0.01 \text{ mg Kg}^{-1}$ ), while AsB and DMA levels were  $< \text{LOQ}$ . Experimental studies have shown that arsenic can be accumulated from water, food or sediment (Maher and Butler, 1988). The isopod *E. spinigera* is a benthic species living mostly on marine sediments, which are the largest geochemical reservoir of arsenic, containing in excess of 99.9% of the element (Maher and Butler, 1988). Strong correlations of the concentration of As in tissues of benthic organisms and in sediments has demonstrated the ability of organisms to use a fraction of particulate-bound arsenic; both arsenic (V) and arsenic (III) are found in the interstitial waters of sediments and bacterial reduction may mediate the redox chemistry of arsenic in sediments (Maher and Butler, 1988). We therefore suggest that the different As levels between the two carnivorous invertebrates, *Flaccisagitta enflata* and *Euridyce spinigera* are due to a different exposure to different habitats, i.e. water columns and sediments, and the presence of iAs in isopods may be related to its benthic habit, living in close association with sediments.

## CONCLUSIONS

The widespread development and application of bioindicators has been in place since the 1960s, and bioindicators are commonly used because environmental practitioners need cost-effective tools that are easy to measure and which provide results that can be clearly communicated to decision makers.

The effectiveness of isopods as excellent bioindicators and bioaccumulators of heavy metals in biomonitoring programs is supported by scientific literature (Longo *et al.*, 2013; García-Hernández *et al.*, 2015), since they are abundant and widely distributed. Soft-bodied forms such as chaetognaths are also important members of the zooplankton for which comparable information is largely lacking. The analyzed species share the same feeding behavior as they are exclusively predaceous, and are at the top of the zooplanktonic food web. However, the fact that they have



459 different habitats- one is hyperbenthic and the other neritic- greatly affects metal bioaccumulation,  
460 as shown in this investigation.

461 The overall objective of bioindicators is to assess the quality of an environment and how it changes  
462 over time, but the use of a single species may represent an oversimplification of a complex system.  
463 Nonetheless, as recently pointed out by Siddig and coauthors (2016), a considerable number of  
464 studies used only a single species to monitor ecosystem changes and quality, and this proportion is  
465 increasing over time. Our results support the consideration that no single species can adequately  
466 indicate the presence of metals or other contaminants in an ecosystem. Depending upon the specific  
467 environment, appropriate bioindicator species or groups of species must be selected. Moreover, in  
468 the perspective of utilizing marine organisms as bio-indicators of metal transfer through the marine  
469 web chain, it is crucial to consider both their habitat and feeding behavior.

470

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476

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698

## 699 **LEGENDS**

### 700 **Fig. 1**

701 Ligurian Sea (Western Mediterranean): sampling site

### 702 **Fig. S1**

703 Trace elements in marine seawater (log scale)

### 704 **Fig. 2**

705 Box-plot diagrams of essential trace elements (mean concentrations  $\pm$  SD) in *F. enflata* and *E.*  
706 *spinigera*. Metal levels are expressed in  $\text{mg kg}^{-1}$  wet weight (Y axis).

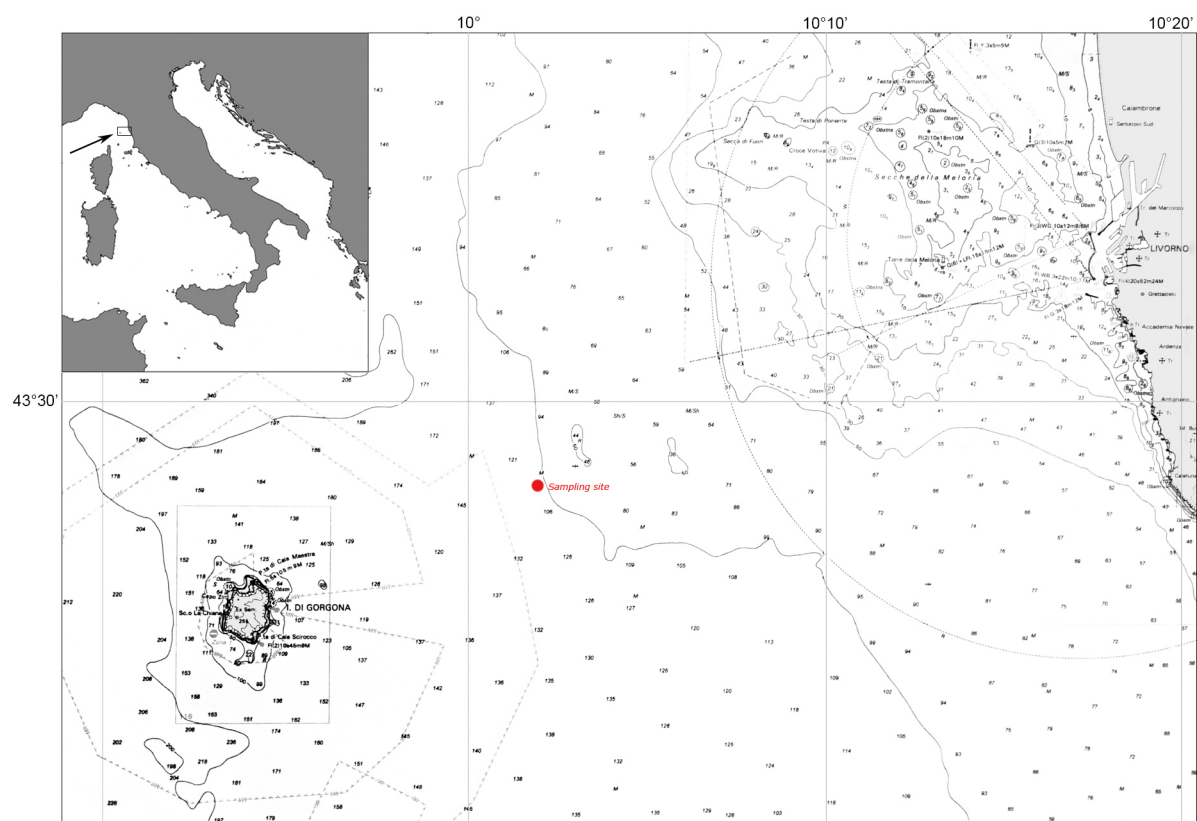
### 707 **Fig. 3**

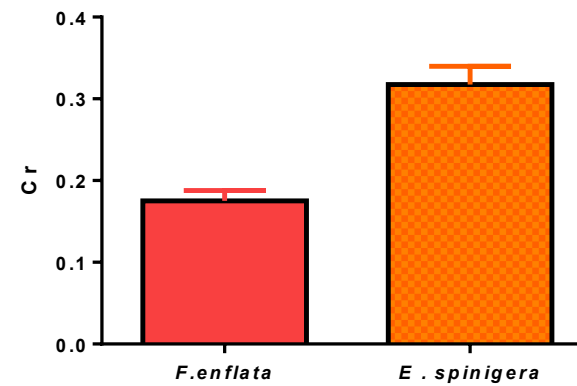
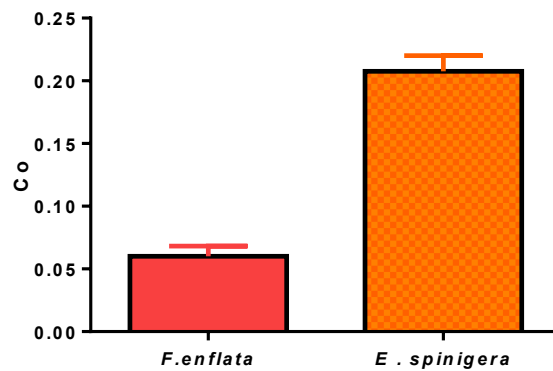
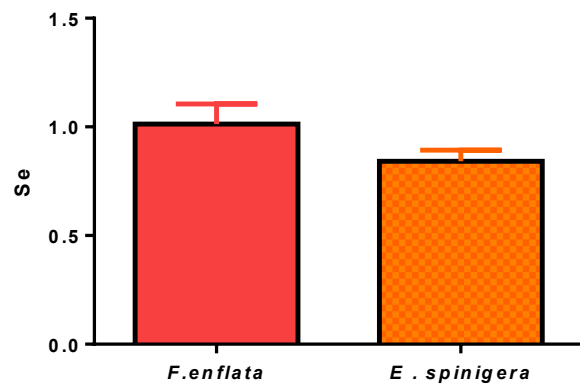
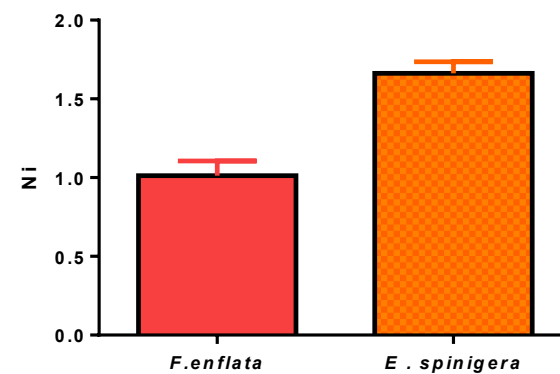
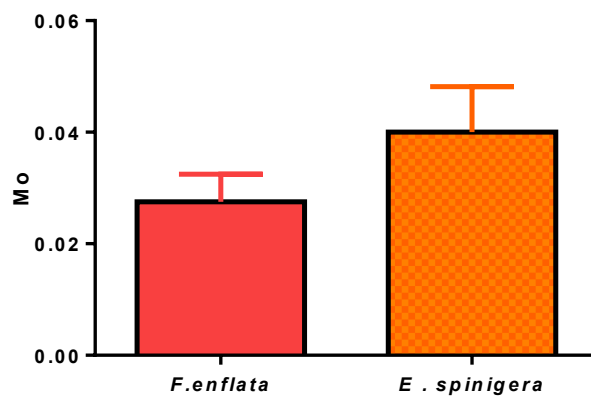
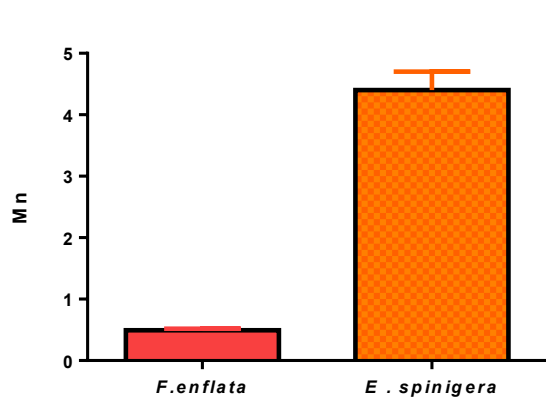
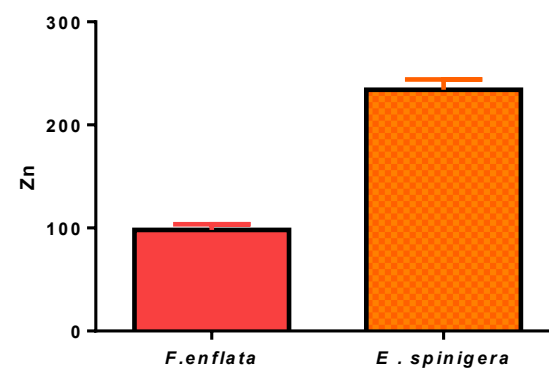
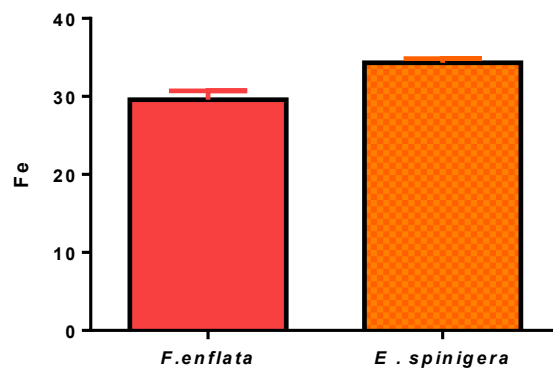
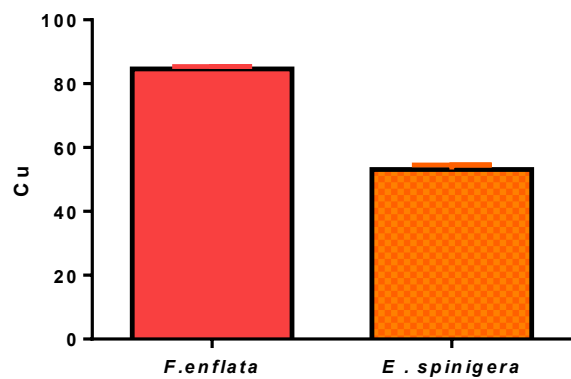
708 Box-plot diagrams of nonessential trace elements (mean concentrations  $\pm$  SD) in *F. enflata* and *E.*  
709 *spinigera*. Metal levels are expressed in  $\text{mg kg}^{-1}$  wet weight (Y axis).

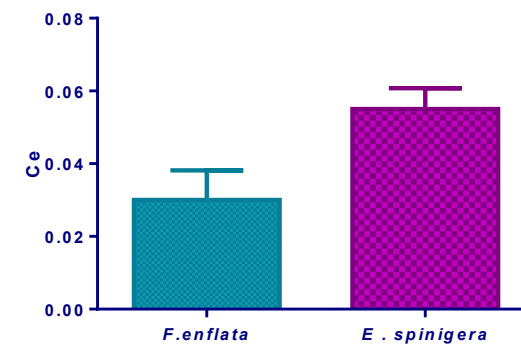
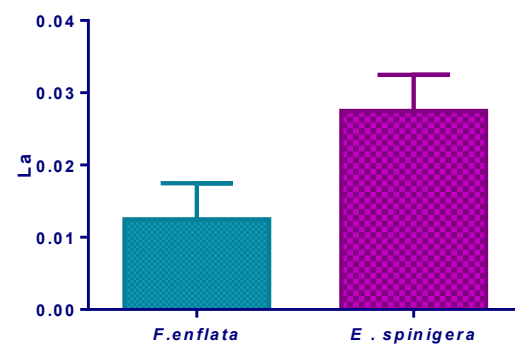
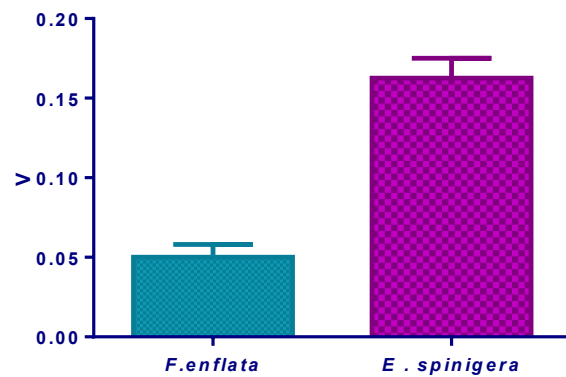
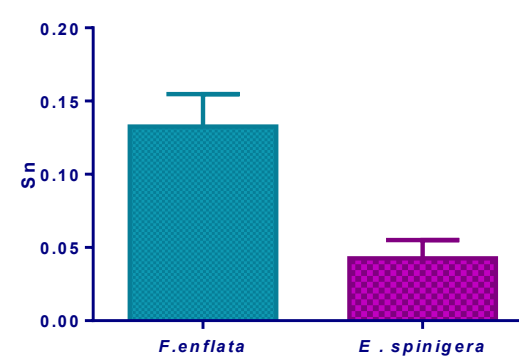
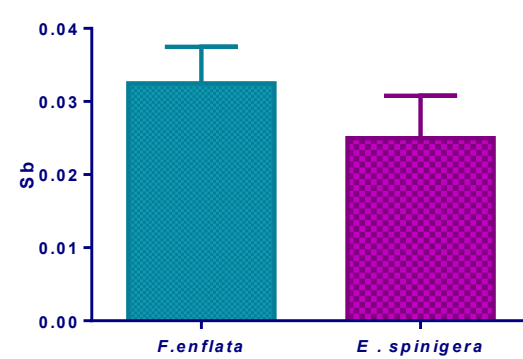
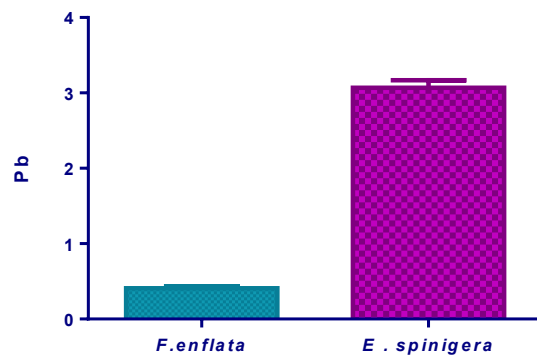
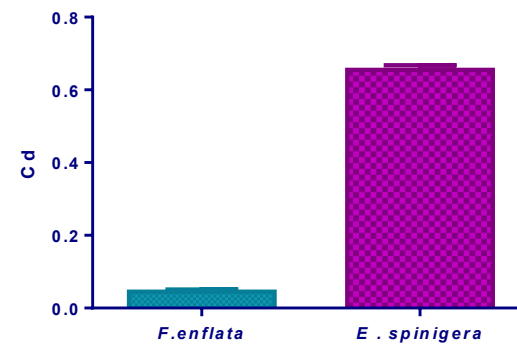
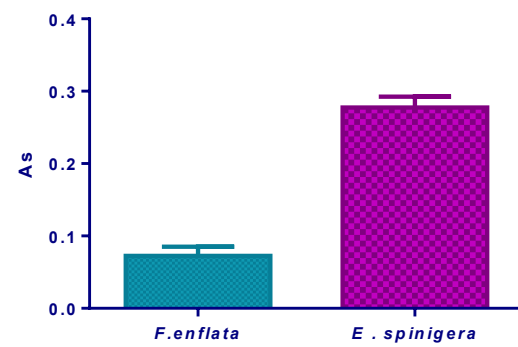
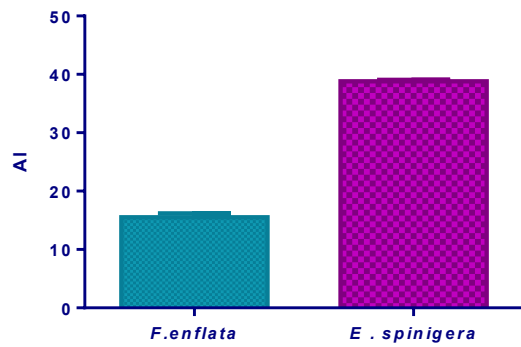
### 710 **Fig. 4**

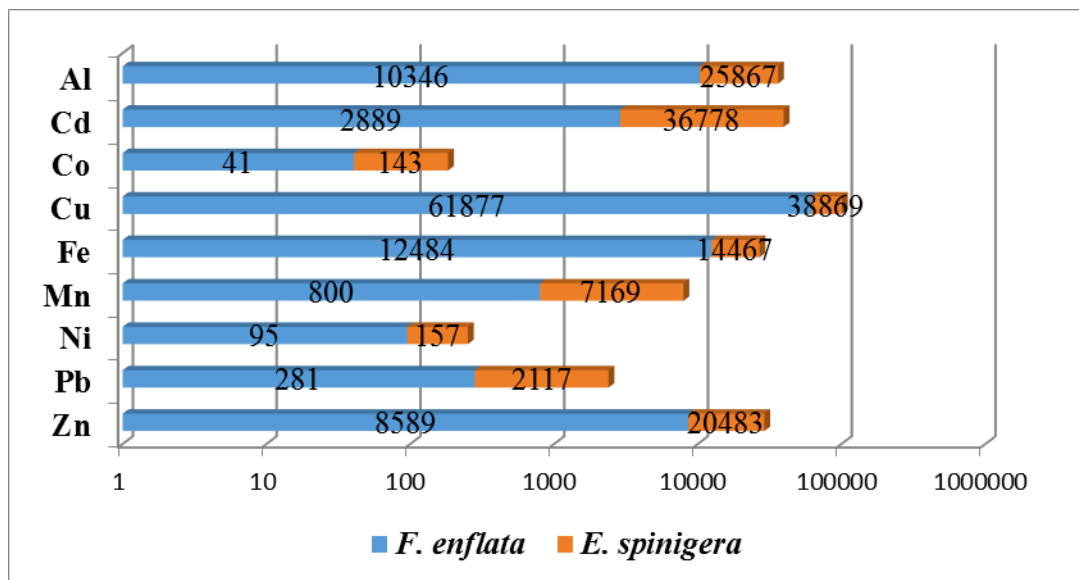
711 Bioaccumulation factors (BAFs) in *F. enflata* and *E. spinigera*.

712









**Table 1**  
**Trace elements (mg Kg<sup>-1</sup> w.w.) in the chetognat *F. enflata* and in the isopod *E. spinigera***

		<b>sample 1</b> <b>(n= 618</b> <b>individuals)</b>	<b>sample 2</b> <b>(n= 602</b> <b>individuals)</b>	<b>sample 3</b> <b>(n= 576</b> <b>individuals)</b>	<b>sample 4</b> <b>(n= 603</b> <b>individuals)</b>	<b>mean</b>	<b>SD</b>	<b>min</b>	<b>max</b>
<b><i>Flaccisagitta enflata</i></b>	<b>Al</b>	14.53	15.65	15.87	16.02	15.52	0.72	14.53	15.87
	<b>As</b>	0.09	0.07	0.07	0.06	0.07	0.01	0.07	0.09
	<b>Cd</b>	0.04	0.05	0.04	0.05	0.05	0.01	0.04	0.05
	<b>Ce</b>	0.03	0.02	0.03	0.04	0.03	0.01	0.02	0.03
	<b>Co</b>	0.05	0.06	0.07	0.06	0.06	0.01	0.05	0.07
	<b>Cr</b>	0.16	0.19	0.17	0.18	0.18	0.02	0.16	0.19
	<b>Cu</b>	84.81	85.64	83.95	83.98	84.60	0.85	83.95	85.64
	<b>Fe</b>	30.91	30.02	28.33	29.12	29.60	1.31	28.33	30.91
	<b>La</b>	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02
	<b>Mn</b>	0.52	0.51	0.48	0.46	0.49	0.02	0.48	0.52
	<b>Mo</b>	0.03	0.03	0.02	0.03	0.03	0.01	0.02	0.03
	<b>Ni</b>	0.98	1.13	1.03	0.91	1.01	0.08	0.98	1.13
	<b>Pb</b>	0.39	0.42	0.44	0.39	0.41	0.03	0.39	0.44
	<b>Sb</b>	0.04	0.03	0.03	0.03	0.03	0.01	0.03	0.04
	<b>Se</b>	0.39	0.38	0.36	0.35	0.37	0.02	0.36	0.39
	<b>Sn</b>	0.11	0.12	0.14	0.16	0.13	0.02	0.11	0.14
	<b>V</b>	0.06	0.04	0.05	0.05	0.05	0.01	0.04	0.06
	<b>Zn</b>	100.02	99.97	8.99	102.51	98.12	5.78	89.99	100.02
		<b>sample 1</b> <b>(n= 297</b> <b>individuals)</b>	<b>sample 2</b> <b>(n= 303</b> <b>individuals)</b>	<b>sample 3</b> <b>(n= 305</b> <b>individuals)</b>	<b>sample 4</b> <b>(n= 328</b> <b>individuals)</b>	<b>mean</b>	<b>SD</b>	<b>min</b>	<b>max</b>
<b><i>Eurydice spinigera</i></b>	<b>Al</b>	39.06	38.44	38.95	38.76	38.80	0.33	38.44	39.06
	<b>As</b>	0.26	0.29	0.29	0.27	0.28	0.02	0.26	0.29
	<b>Cd</b>	0.64	0.67	0.65	0.66	0.66	0.02	0.64	0.67
	<b>Ce</b>	0.05	0.05	0.06	0.06	0.06	0.01	0.05	0.06
	<b>Co</b>	0.19	0.22	0.21	0.21	0.21	0.02	0.19	0.22
	<b>Cr</b>	0.34	0.33	0.29	0.31	0.32	0.03	0.29	0.34
	<b>Cu</b>	53.25	55.08	51.89	52.38	53.15	1.60	51.89	55.08
	<b>Fe</b>	33.69	33.98	34.67	34.87	34.30	0.50	33.69	34.67
	<b>La</b>	0.02	0.03	0.03	0.03	0.03	0.01	0.02	0.03
	<b>Mn</b>	4.41	4.69	3.99	4.52	4.40	0.35	3.99	4.69
	<b>Mo</b>	0.03	0.04	0.05	0.04	0.04	0.01	0.03	0.05
	<b>Ni</b>	1.63	1.75	1.69	1.58	1.66	0.06	1.63	1.75
	<b>Pb</b>	2.99	3.21	3.01	3.06	3.07	0.12	2.99	3.21
	<b>Sb</b>	0.02	0.03	0.02	0.03	0.03	0.01	0.02	0.03
	<b>Se</b>	0.90	0.87	0.79	0.81	0.84	0.06	0.79	0.90
	<b>Sn</b>	0.06	0.03	0.04	0.04	0.04	0.02	0.03	0.06
	<b>V</b>	0.18	0.15	0.16	0.16	0.16	0.02	0.15	0.18
	<b>Zn</b>	245.71	235.08	221.32	233.98	234.02	12.23	221.32	245.71

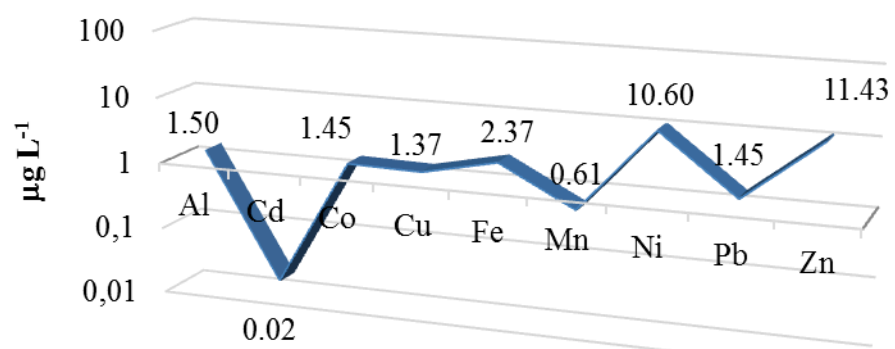
**Table 2. Unpaired t test, comparison between *F.enflata* and *E.spinigera***

<b>Trace element</b>	<b>P value</b>	<b>Summary of P values</b>
<b>Al</b>	P < 0.0001	****
<b>As</b>	P < 0.0001	****
<b>Cd</b>	P < 0.0001	****
<b>Ce</b>	P = 0.0025 (P < 0.01)	**
<b>Co</b>	P < 0.0001	****
<b>Cr</b>	P < 0.0001	****
<b>Cu</b>	P < 0.0001	****
<b>Fe</b>	P < 0.0001	****
<b>La</b>	P = 0.0054 (P < 0.05)	**
<b>Mn</b>	P < 0.0001	****
<b>Mo</b>	P < 0.0041 (P < 0.05)	*
<b>Ni</b>	P < 0.0001	****
<b>Pb</b>	P < 0.0001	****
<b>Sb</b>	P = 0.0972 (P > 0.05)	NS
<b>Se</b>	P = 0.0259 (P < 0.05)	*
<b>Sn</b>	P = 0.0011 (P < 0.05)	**
<b>V</b>	P < 0.0001	****
<b>Zn</b>	P < 0.0001	****

\* Significant at the 0.05 probability level

NS not statistically significant





## Figure captions

### Figure 1

Ligurian Sea (Western Mediterranean): sampling station

### Figure 2

Box-plot diagrams of essential trace elements (mean concentrations  $\pm$  SD) in *F. enflata* and *E. spinigera*. Metal levels are expressed in  $\text{mg kg}^{-1}$  wet weight (Y axis).

### Figure 3

Box-plot diagrams of nonessential trace elements (mean concentrations  $\pm$  SD) in *F. enflata* and *E. spinigera*. Metal levels are expressed in  $\text{mg kg}^{-1}$  wet weight (Y axis).

### Figure 4

Bioaccumulation factors (BAFs) in *F. enflata* and *E. spinigera*.